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Compositional Changes of Douglas Fir Seeds During Germination[1]

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Summary. Changes in weight, water content, nucleic acids, nucleotides, carbohydrates, lipids, nitrogenous and phosphorus compounds in embryo and gametophyte of Douglas fir seeds (*Pseudotsuga menziesii* Franco) were studied at 6 stages of germination. Lipids, proteins, and reserve phosphorus compounds in the gametophyte were utilized for the synthesis of carbohydrates, structural components, and soluble compounds in the seedling.

The general quantitative metabolic changes that occur during germination of Douglas fir seeds are comparable to those known for angiosperm seeds.

Seeds of gymnosperm species include haploid storage tissue, the female gametophyte and diploid embryo (1), whereas the seeds of angiosperms usually have diploid cotyledons or perisperm, or triploid endosperm as storage sites. Compositional changes associated with germination in angiosperm seeds are well documented (14, 18) but only fragmentary studies have been conducted with gymnosperm seeds (3, 4, 12). An investigation of important structural components and intermediate metabolites during germination of gymnosperm seeds would provide valuable comparative biochemical information and a background for further cellular and molecular biological studies of germination processes.

Data presented in this paper indicates that the metabolic events of germination in gymnosperms are similar to those characteristic for angiosperm seeds. Apparently the multiplication of chromosome number in storage organs has no specific effect on the synthesis of enzymes required for degradation and utilization of storage chemicals. Embryo growth seems to follow a general metabolic pattern regardless of taxonomic position.

Materials and Methods

Materials. Seeds of Douglas fir (*Pseudotsuga menziesii* Franco) were collected from a single tree in Corvallis in 1964, processed by screening and air blowing to an average weight of 15 mg and a water content of 5 %, then were stored at 3° in an airtight container. Seeds were stratified for four weeks at 3° prior to germination. They were then germinated on Spongerok in a plastic box at 30° during the 8-hour photoperiod and at 20° during the dark period. Six stages were arbitrarily chosen for study: A) air-dried; B) stratified seed; C) radicle emerging; D) radicle elongating; E) cotyledon emerging; and F) cotyledons elongating and plumule emerged. The average times of incubation required for attainment of stages C, D, E, and F were 5, 7, 10, and 14 days, respectively.

Weight, Water Content and Total N and P of Seed Parts. Four replications of 10 seeds from each of the 6 growth stages were separated at 3° into seed coat, embryo or seedling, and gametophytic tissue. Fresh weights were recorded, and their dry weights and water content were determined by drying at 85° for 16 hours. Total nitrogen content of the separated seed parts was estimated by the micro-Kjeldahl procedure. Total phosphorus content was determined by the Fiske-Subbarow method (8) after wet washing with sulfuric acid and H_2O_2.

Determination of Nucleic Acids and Nucleotides. Four replications of 20 separated gametophytes and embryos or seedlings were used for each stage. The procedures of Ingle et al. (10) for estimating nucleic acids and soluble nucleotides were followed. Highly purified yeast RNA, highly polymerized calf thymus DNA, and AMP (all from Sigma) were carried through the procedures, and were used as standards for quantitative estimation.

Determination of Other Chemical Constituents. Seeds of the 6 stages were separated and directly dropped into a known weight of ice-cold methanol. Approximately 4 g of the first 2 stages, 15 g of stages C and D, and 30 g of stages E and F were separated and used for subsequent analysis. The weight of each separated part was obtained by subtracting the weight of methanol from the total

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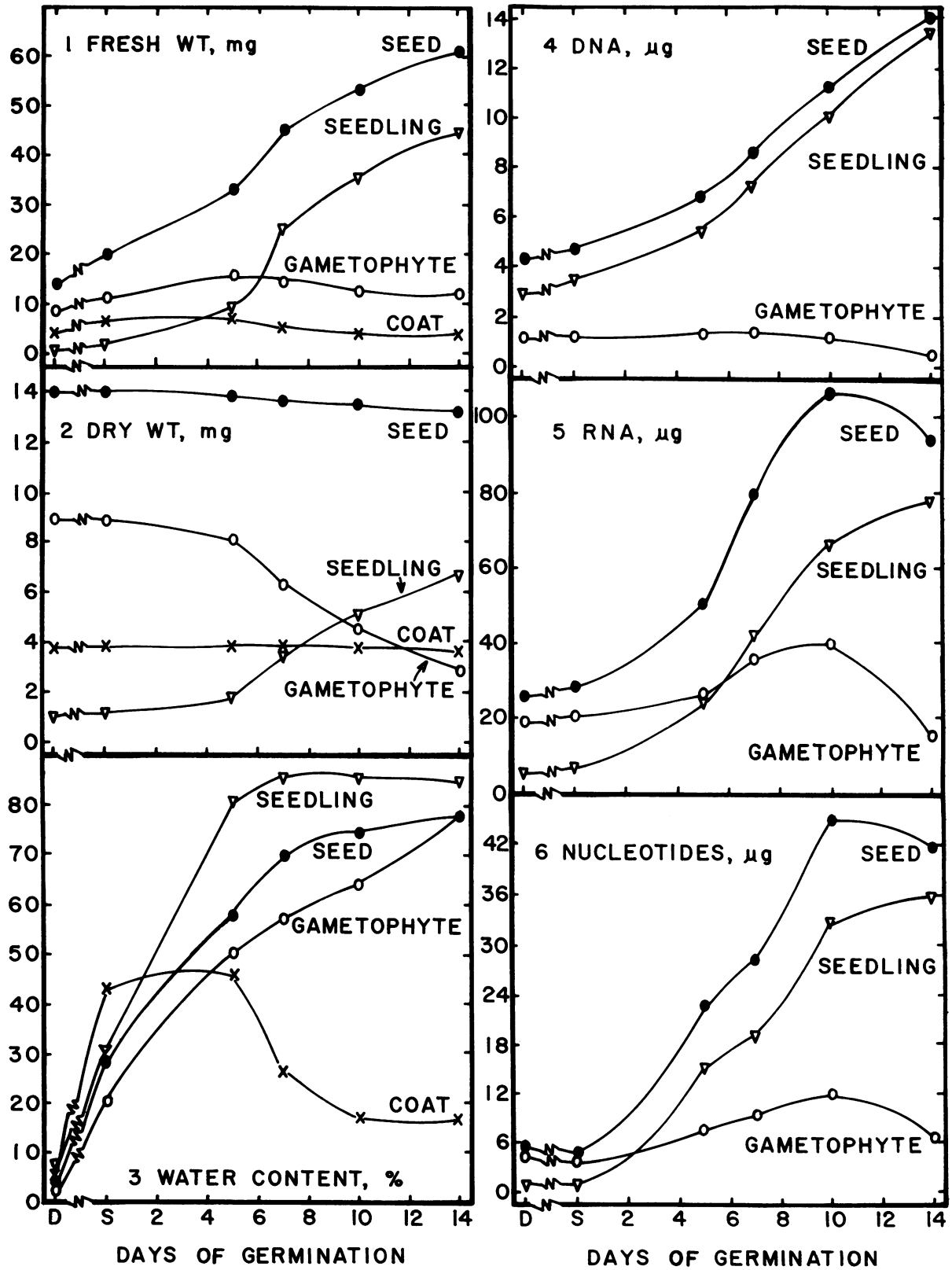


FIGS. 1–6. The changes of fresh weight, dry weight, water content, DNA, RNA, and nucleotides in embryo, female gametophyte, seed coat and whole seed of Douglas fir seed during germination. D = air dried seed, S = stratified seed.

weight of methanol and the material. The material was ground in methanol, in the ratio of 1:10 (wt:vol) at high speed for 10 minutes in a Waring Blendor; then 2 volumes of chloroform were added to the homogenate, the mixture was kept at room temperature for 2 hours to extract lipids. The mixture was filtered, and the extraction repeated twice. The 3 extracts were combined and washed with 2 volumes of water in a separatory funnel. The white emulsion at the interphase was centrifuged at 5000 × *g* for 10 minutes at 20° and the concentrated emulsion combined with the upper organic phase and dried first in a vacuum evaporator, then in a vacuum oven at 50° for 24 hours to obtain the weight of total lipids (9). The aqueous washings were combined with the alcohol extract described below for the analysis of soluble metabolites.

The chloroform-methanol extracted residue was dried at 60° in vacuo for 16 hours and then weighed. One hundred mg of the residue was extracted with 10 ml 80 % (v/v) ethanol in an Omnimixer for 10 minutes at 6500 rpm. The homogenate was filtered and the extraction repeated twice. The 3 extracts were combined with a proportional aliquot of the lipid-washing obtained above and considered as the total soluble fraction. Dry weight in the soluble fraction was determined in an aliquot by drying at 60° in vacuo. Soluble N and P were determined in additional aliquots by the micro-Kjeldahl, and Fiske-Subba Row method, respectively. The remaining soluble fraction was concentrated in a vacuum evaporator, dissolved in water, filtered through a mat of celite and made up to 100 ml. Sugars, amino acids and P were estimated in the aqueous soluble fraction by the anthrone method (19), ninhydrin method (5) and Fiske-Subbarow method, respectively.

The ethanol extracted residue was dried in vacuo at 60° for 16 hours and weighed as insoluble residue. Fifty mg of the residue was hydrolyzed in 1 N HCl for 1 hour by refluxing. The mixture was filtered and starch and other easily hydrolyzable carbohydrates were determined in the filtrate by the anthrone method using glucose as standard and applying a correction factor of 0.9.

All values reported are means based on 4 replicated determinations each.

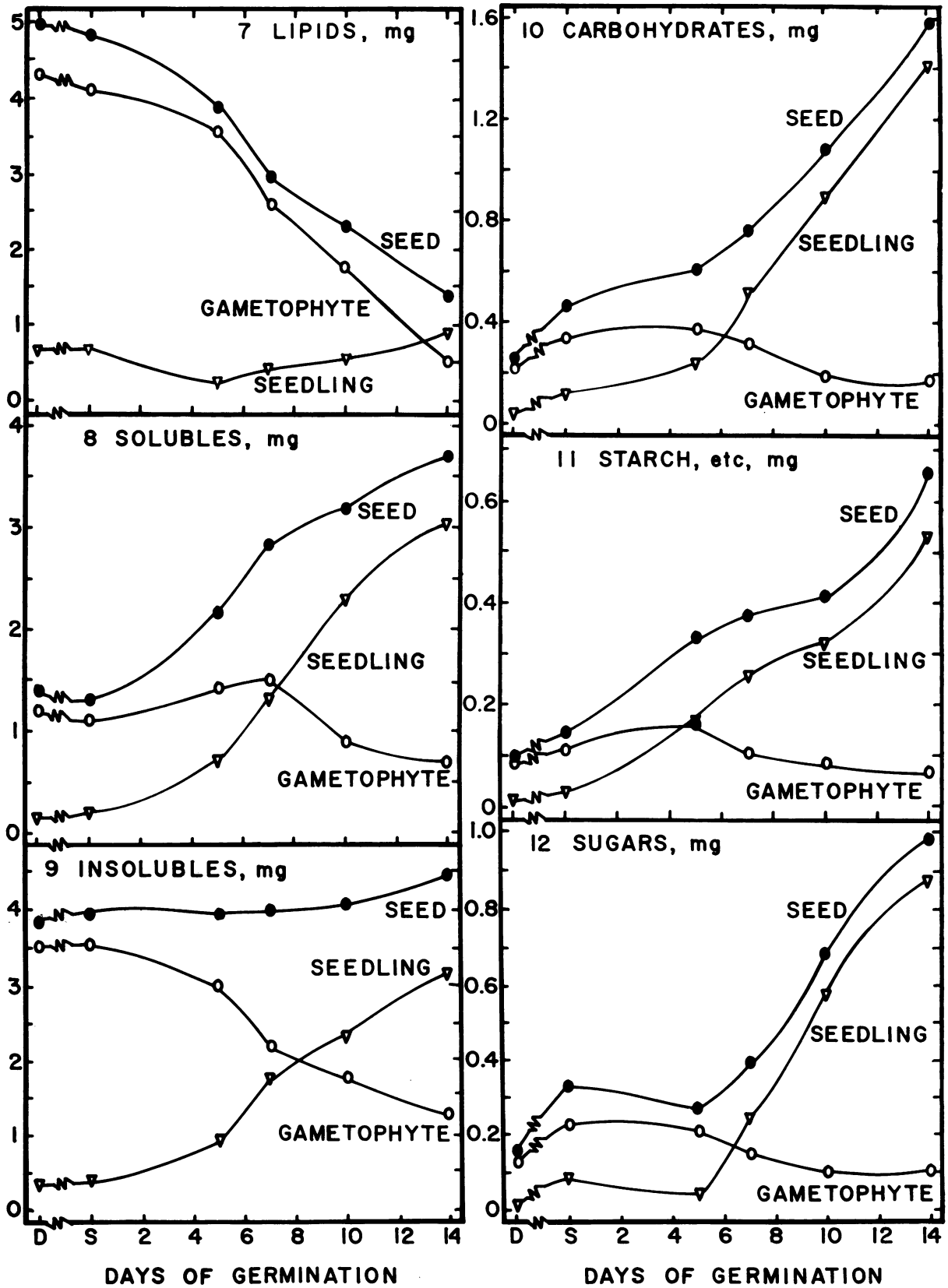
Results and Discussion

Germination is indeed a growth process of the embryo as shown by the 35-fold increase of fresh weight and 6-fold dry weight increase of the seedling, whereas little change of fresh weight and a 70 % reduction of dry weight were observed in gametophytic tissue (fig 1, 2). Apparently the embryo grew at the expense of the gametophyte with a loss of 8 % of the total weight, probably for energy supply (fig 2). The seed Coat seemed to assist in water uptake during the early stages of germination (fig 3). The differential degree of hydration as seen from the water content in seedling and gametophyte affected their metabolic activity during germination. This phenomenom is known in other seeds (10, 15).

Little change of DNA content was observed in the gametophyte during early stages of germination and a 60 % reduction was found at the end of germination (fig 4). The embryo showed a slight increase of DNA during stratification, a doubling of DNA content at the radicle emerging stage, and a 4.5-fold increase toward the end of germination. The DNA content of the gametophyte was very low and constituted only 0.013 % of its dry weight, whereas that of the embryo was 0.25 % (fig 4 and 2). This difference could be attributed to: (a) the haploid nature of the gametophyte; (b) the large quantity of reserve food in the gametophyte; and (c) the doubled DNA content per cell in embryonic tissue prior to germination (17).

In dry seed, 6 μg and 19 μg of RNA were found in the embryo and the gametophyte, respectively (fig 5). These quantities constituted 0.50 and 0.21 % of their dry weight, respectively. A slight increase in RNA in both embryo and gametophyte was found in stratified seed. A rapid rate of RNA synthesis was observed in seedlings and a moderate increase followed by a rapid decrease were observed in gametophyte. At about the time of plumule emergence, seedlings showed a 12-fold increase in RNA while the gametophyte contained only two-thirds of its original RNA content. The sigmoid curve of RNA increase in seedlings interestingly paralleled fresh and dry weight and DNA increases. This perhaps indicates the accumulation mainly of the structural RNA (ribosomal) and to a lesser extent, the messenger RNA and soluble RNA (2). In the gametophytic tissue, the moderate increase of RNA coincided with the stages of rapid transfer of dry matter from gametophyte to seedling. This increase may reflect an active synthesis of enzymes for rapid degradation of proteins and lipids, and conversion of acetyl CoA to sugars. From ultrastructural changes observed in this tissue, the genesis of plastids and increase of mitochondria also might account for some of the increase (6). At the end of germination, gametophyte apparently ceased its function of nourishing the seedling by hydrolyzing RNA to nucleotides and presumably transferring them to seedling. The hydrolysis of RNA and DNA at this stage of development in gametophytic tissue was further verified by the marked increase of soluble P and P_i (fig 17, 18).

The change in soluble nucleotides was similar to that of RNA except for a small reduction shown by the gametophyte after stratification (fig 6). This reduction perhaps provides substrate for the small increase in DNA in the embryo and in RNA in both embryo and gametophyte during stratification. A 24-fold increase of nucleotides in seedling at the end of germination was observed, while only a 2-


7 LIPIDS, mg
SEED
GAMETOPHYTE
SEEDLING
8 SOLUBLES, mg
SEED
SEEDLING
GAMETOPHYTE
9 INSOLUBLES, mg
SEED
SEEDLING
GAMETOPHYTE
10 CARBOHYDRATES, mg
SEED
SEEDLING
GAMETOPHYTE
11 STARCH, etc, mg
SEED
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12 SUGARS, mg
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fold increase in gametophyte at the cotyledon emerging stage was found. These changes in DNA, RNA and soluble nucleotides indicated de novo synthesis of nucleic acids and are in general agreement with the findings on corn, peanuts, and castor bean (2, 11, 14).

Lipids were the major food reserve in both gametophyte and embryo in dry seed (fig 7). They composed 48 and 55 % of the dry weight of the gametophyte and embryo, respectively. During stratification, gametophytic lipids were reduced by 0.3 mg but little change was shown in the embryo. During germination, lipids in the gametophyte continued to decrease from 4.1 to 0.5 mg. After an initial drop to 0.3 mg, the lipid content of the seedling gradually increased to 0.9 mg. The analysis of lipids isolated from these 2 tissues at various stages of germination has been undertaken and will be reported in a separate paper.

The change of metabolites soluble in aqueous methanol and ethanol during germination is summarized in figure 8. The embryo had as soluble compounds 13 % of its dry weight, a slight increase during stratification and a rapid increase to 45 % of seedling dry weight, toward the end of germination. In the gametophyte soluble compounds constituted 14, 12, 24 and 24 % of the dry weight at each growth stage, respectively. The data for the residue insoluble in chloroform-methanol, aqueous methanol, and ethanol are plotted in figure 9. A rapid accumulation of insoluble residue in the seedling coincided with a marked reduction in the gametophyte after radicle emergence.

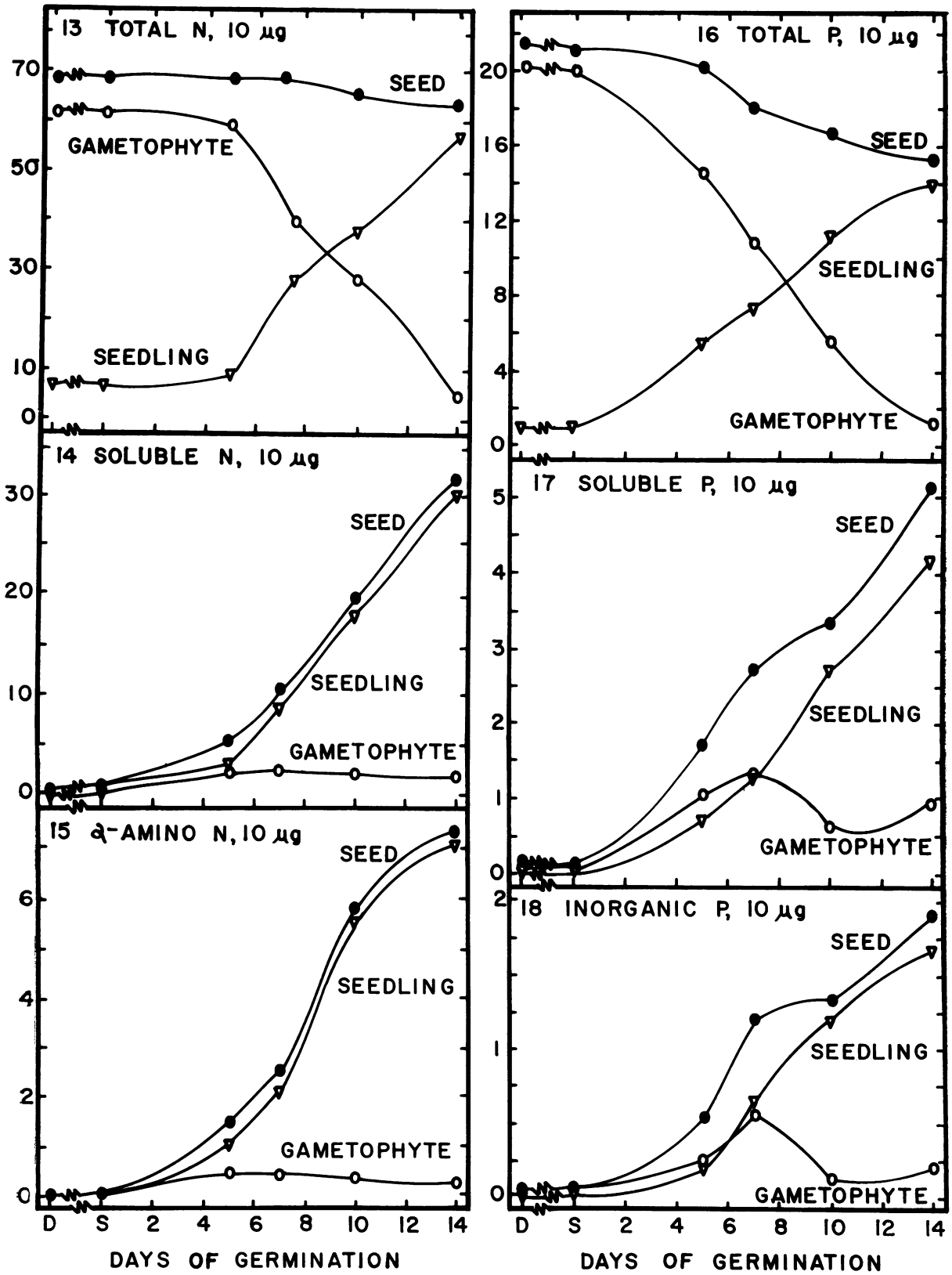
Figures 7, 8, and 9 show that during germination lipids and insoluble residues in gametophytic tissue were utilized for the development of the embryo. Further electron microscopic study of the ultrastructural changes during germination, cytochemical staining, separation of organelles with differential centrifugation, and chemical separation and characterization indicated that reserve lipids were stored in fat bodies, and insoluble proteins conjugated with lipids were located in protein bodies. These fat and protein bodies occupied 85 % of the volume of ungerminated gametophytic tissue and constituted about 90 % of its weight (6). Detailed electron microscopic and organellar studies will be reported in subsequent papers. As germination proceeded, the storage organelles decreased in number and weight, undergoing structural as well as biochemical changes. These changes resulted in an increase of soluble intermediates. By trichloroacetic acid precipitation, ion exchange resin column chromatography and paper chromatography, sugars, sugar nucleotides, other organic phosphates, P_i, amino acids, amides, peptides, soluble proteins, organic acids, etc. were detected in the soluble fraction. A detailed qualitative and quantitative examination of these intermediates would be of interest but was not attempted.

Reserve fats in seeds usually are converted to sugars via the glyoxylate cycle, then transported to the growing seedling for further utilization (15, 16). Changes of carbohydrates were therefore, followed in seed tissues during germination. Figure 10 shows a slow accumulation of total carbohydrates during stratification, a plateau prior to radical emergence, then a rapid increase. At early stages of germination, the increase was in both embryo and gametophyte, and a rapid increase was observed in seedling at the later stages (fig 10, 11, 12). These sequential changes agree with the findings in angiosperm and verify the means of food transfer during seed germination (15, 18). A pronounced increase of sugars in both embryo and gametophyte after stratification was of interest and may be considered a beneficial effect of stratification.

Utilization of nitrogenous reserves is shown in figures 13 to 15. A slight reduction (9 %) of total N was observed, probably due from root diffusional losses (12). A rapid quantitative transfer from gametophyte to seedling is accomplished by soluble compounds with little accumulation in storage site. Solvation of insoluble protein, activation of preexisting enzymes, degradation of storage protein, active transport and synthesis to new protein, and other nitrogenous compounds are apparently a chain of events occurring mainly in the gametophyte for the first 3 phases and in the seedling for the last one. It is also interesting to note that soluble nitrogenous compounds in the seedling comprised about 50 % of the total nitrogenous compounds of which only 10 to 15 % were free amino acids and amides. These changes agree with those reported for corn and beans and are apparently of the general trend for seed germination (10, 12, 15, 16).

Phosphorus compounds are important in the dynamic processes of synthesis and energy supply. The changes during germination are summarized in figures 16 to 18. A reduction of 29 % in total phosphorus in seed at the end of germination is puzzling, and perhaps it could be attributed to root diffusion. Soluble phosphorus compounds in seed increased 40-fold and constituted 30 % of the total P at the end of germination with a differential distribution of 80 % in the seedling and 20 % in the

FIGS. 7–12. The changes of lipids, total aqueous-alcohol soluble compounds, insoluble residues, total carbohydrates, starch and sugars in embryo, female gametophyte, and seed without coat of Douglas fir during germination. D = air dried seed, S = stratified seed.



FIGS. 13–18. The changes of total nitrogenous compounds, soluble nitrogenous compounds and α-amino N-compounds; total, soluble, and inorganic phosphorus compounds in embryo, female gametophyte and seed without coat of Douglas fir during germination. D. = air dried seed, S = stratified seed.

gametophyte. Inorganic phosphate increased similarly to the soluble phosphorus compounds, and constituted about 30 to 50 % of the soluble P compounds. The reserve form of phosphorus compound probably is phytin since it is commonly found in angiosperm seed (15). The change of phosphorus compounds during germination is in general agreement with cotton seed (15).

Generally, the metabolic changes during germination in Douglas fir seeds are similar to angiosperm seeds. The chain of events involve degradation of reserve lipids, proteins, and phosphorus compounds and the transport of these compounds to the embryo for synthesis of cellular components. There is a unique feature of physical separation and genetic difference of gametophyte tissue and embryo in this material. This feature and the data presented in this paper suggest that the gametophyte may provide a system with less complication of the simultaneous catabolic and anabolic activities and thus suitable for studies involving A) lipolysis, B) synthesis of glyoxylate cycle enzymes, C) activation of pre-existing enzymes and messenger RNA (6), D) conformational changes in insoluble reserve proteins to active forms or the mechanism of protein synthesis (18), and E) isolation and degradative metabolism of reserve phosphorus compounds.

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